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Surface coat proteins of the pine wood nematode, *Bursaphelenchus xylophilus*: profiles of stage and isolate specific characters

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Summary – The present study was made to determine the binding patterns of several lectins to the surface coat (SC) proteins of various isolates and developmental stages of the pine wood nematode (PWN), *Bursaphelenchus xylophilus*. Also, the detailed characteristics of the SC proteins were profiled by using molecular techniques. The lectin-binding study demonstrated the stage-specific characters of SC in binding to the lectin, wheat germ agglutinin (WGA). WGA-binding was observed only to the outer surfaces of 3rd-stage propagative juveniles and to the egg shells, and this occurred more frequently in virulent than in avirulent PWN isolates. A greater variety of lectins bound to eggs than to any other life stage. For characterization, the SC proteins extracted were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by lectin blotting. The results showed that the carbohydrate and protein patterns of the SCs of the PWN changed during nematode development.

Keywords –SDS-PAGE, lectin blot, glycoprotein, glycan, WGA.

Pine wilt disease, caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is one of the most serious forest diseases in East Asia and it has been found in Portugal (Mota *et al*., 1999). PWN has both a propagative and a dispersal form; with the latter being carried from dead to healthy pine trees by the beetle vector, *Monochamus* spp., i.e. spread of the disease occurs via the 4th-stage dispersal juvenile (DJ_{IV}) (Kobayashi *et al*., 1984).

The pathology of pine wilt disease has been intensively studied (*e.g.*, Mamiya, 1983), and various hypotheses have been advanced regarding the exact mechanisms of pathogenicity. For example, cell-wall-degrading enzymes including cellulases and pectate lyases secreted by the PWN have been shown to trigger the development of early disease symptoms (Odani *et al*., 1985; Kikuchi *et al*., 2004, 2006; Zhang *et al*., 2006; Jones *et al*., 2008). Although these cell-wall-degrading enzymes are probably involved in the interaction of PWN with its host, Jones *et al*. (2008) described that these enzymes are not the sole causes of pathogenicity.

Nematode surface coat (SC) is considered to play an important role in host–parasite interactions as well as secretion products including cell-wall-degrading enzymes (Spiegel & McClure, 1995; Sharon *et al*., 2002), and the importance of the SC is thought to lie in its dynamic nature. The SCs of nematodes are usually located

external to the epicuticle, and mostly they are glycoproteins (Blaxter & Robertson, 1998). Many species of nematodes shed and regenerate their SC (Blaxter & Robertson, 1998), and most of those which have been studied in detail were animal-parasitic nematodes (Maizels & Loukas, 2001). Compared to animal-parasitic and free-living nematodes, the characteristics of the SC of plant-parasitic nematodes are not as well known (Lopez de Mendoza *et al*., 1999; Blaxter & Bird, 1997). Among the plant-parasitic nematodes, the SCs of the root-knot nematodes, *Meloidogyne* spp., have been well studied and found to have an important biological role with the SC of plant-parasitic nematodes appearing to be multifunctional, that is, involved in adhesion, lubrication, and modulation to help counter host defense responses (Bird, 2004; Gravato-Nobre *et al*., 1999). Also, carbohydrates of the SC have been suggested to be a specific elicitor triggering host defense responses (Spiegel & McClure, 1995; Gravato-Nobre & Evans, 1998).

To date some reports suggest that host defense responses play a key role in pine wilt symptom development (Iwahori & Futai, 1993; Yamada, 2008). In general, the defense responses of higher plants are initiated when the plant detects invasion of pathogens, more precisely, their elicitors, usually derived from the surface components of or the molecules secreted by the pathogens. Since the SC of PWN directly contacts

the cell surface of the host pine, the SC should deserve more attention in determining the early interaction of the PWN and pine tree, which results in subsequent host defense responses.

The first goal of the present study was to: (i) profile the SCs of the PWN by labeling them with several lectins to investigate differences in carbohydrate profile in the various life stages and isolates of PWN and (ii) characterize the SC proteins and their glycosylation of the PWN by using molecular techniques.

Materials and methods

NEMATODES

Five isolates of *Bursaphelenchus xylophilus*, *i.e.*, three virulent isolates (S10, T-4, and Troia) and two avirulent isolates (OKD-1 and C14-5), were used in the lectin-labeling study. A mixed culture of the propagative forms including 2nd-stage juvenile (J2), 3rd-stage juvenile (J3), 4th-stage juvenile (J4) and adult, and egg were propagated on the fungus *Botrytis cinerea* (Fr.) Pers. growing on autoclaved barley grains at 25°C in 50-ml Erlenmeyer flasks. After 2-week incubation, a few barley grains

were picked up and put on a sheet of paper floating on distilled water in a 6-cm diameter petri dish, and then, after 30 min-incubation at room temperature, the PWNs which emerged from the grains into the water were immediately used in each test.

The virulent S10 isolate was used for surface coat extraction. After rearing the nematodes on *B. cinerea* growing on barley grains at 25°C for 5 days, the nematode eggs were collected from the culture (Iwahori & Futai 1985) and incubated in phosphate buffered saline (PBS, pH 7.4) at 25°C. After 2 days, the hatched J2s were collected and transferred onto growing edge of *B. cinerea* on potato dextrose agar (PDA) in a 90-mm diameter petri dish. PWN development was synchronized by allowing eggs to hatch in the absence of food. The PWN J2s were collected 6 h after re-initiation of feeding and then directly used in the following experiments. The J3s, J4s and adults were collected 30 h, 54 h and 78 h after re-initiation of feeding and passed through nylon and polyester mesh sieves (Sefar Holding Inc.) to separate them into their respective stages, i.e. the J3s, J4s and adults were obtained by passing the nematodes through (i) an 11-µm-pore polyester mesh onto a 10-µm-pore nylon mesh, (ii) a 15-µm-pore nylon mesh onto an 11-µm-pore polyester mesh and (iii) a 20-µm-pore nylon mesh onto a 15-µm-pore nylon mesh, respectively. Each PWN stage was used in the following experiment.

The dispersal forms, *i.e.*, 3rd-stage dispersal juveniles (DJ_{III}) and 4th-stage

dispersal juveniles (DJ_{IV}), were prepared according to Togashi (2004). Briefly, five dead pine trees were felled at Keihoku, Ukyo-ku, Kyoto Prefecture, on 25 April 2007 and postdiapause larvae of the PWN vector beetle *M. alternatus* were collected from pupal chambers inside the tree boles. After incubation for 2 months at 10°C, all the *M. alternatus* larvae were placed in 100-ml Erlenmeyer flasks where *B. xylophilus* was being propagated on the fungus *Ophiostoma minus* (Hedgcock) H. & P. Sydow cultured on 10 g of barley grains and 7.5 g of *Pinus densiflora* wood chips, and incubated at 25°C. One week after eclosion, the beetles were crushed with a blender for 1 min, and using the Baermann funnel technique to obtain the DJ_{IV}. DJ_{III} were also extracted from the culture media using the Baermann funnel technique.

LECTIN LABELING

The fluoresceine isothiocyanate (FITC)-conjugated lectins Con A (*Canavalia ensiformis* agglutinin), WGA (*Triticum vulgaris* agglutinin), PNA (*Arachis hypogaea* agglutinin), UEA I (*Ulex europaeus* agglutinin), and RCA_{120} (*Ricinus communis* agglutinin) (all provided by Vector Laboratories, Inc.) were used in the following test (Table 1). The nematodes were washed three times (5 min each time) in PBS before

being fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. The fixed nematodes were rinsed three times in PBS, and incubated with the lectin solutions for 30 min in the dark at room temperature. Con A, WGA, PNA, and RCA_{120} were diluted to 50 µg/ml with PBS, and UEA I was diluted to 20 µg/ml with PBS. All the treatments were followed by three-time washes with PBS, and the nematodes were then mounted on a glass slide, covered with a coverslip, and observed using incident fluorescence microscopy with the filter of an excitation wavelength of 450-490 nm. The microscopic images of the nematodes were recorded with a Zeiss Axiovert 200 microscope equipped with a confocal laser-scanning module (Zeiss LSM510). Serial-section images were acquired and reconstructed into 3D images using the LSM510 operation system and software. The serial images (LSM files) were first transformed into jpeg images and then converted into AVI movie files using the AVI edit (Hasegawa *et al.*, 2006). Thirty nematodes of each stage were observed to check whether or not the outer surface of the nematode was labeled with lectins. The degree of surface labeling of the PWNs at each stage with lectins was classified into five levels according to the proportion of the number of nematodes labeled with each lectin as follows: 0/30; ±, 1/30 to 9/30; +, 10/30 to 19/30; ++, 20/30 to 25/30; and +++, 26/30 to 30/30 (Table 2).

To confirm the specificity of the lectin-labeling, lectin solutions were all incubated with their competitive sugars (Table 1) for 30 min prior being incubated with the nematodes. Specific labeling was indicated by a subsequent reduction in fluorescence when the nematodes were incubated in each lectin solution.

PERIODATE PRETREATMENT

For all nematode stages, periodate pretreatment for oxidative cleavage of carbohydrate was done by incubating the nematodes in 10 mM sodium periodate ($NaIO_4$) in 100 mM sodium acetate buffer (pH 4.5) for 1 h in the dark at 25°C. The nematodes were then washed three times with PBS, and subsequently incubated with five kinds of FITC-conjugated lectins for 30 min using the same procedure as described above.

EXTRACTION OF SURFACE COAT PROTEINS

The extraction of SC proteins was conducted using the procedure described by Spiegel *et al*. (1996, 1997) with some modifications. A mass of the S10 nematodes at

each stage except for dispersal juveniles, *i.e.*, J2: 300,000; J3: 200,000; J4: 150,000; adult: 100,000; egg: 200,000, was suspended in 500 µl of 1% sodium dodecyl sulfate (SDS) in PBS with 1% protease inhibitor cocktail for mammalian cells (Sigma), and gently agitated for 1 h at 25°C. The nematodes were then pelleted by centrifugation at 15,000 × g for 5 min at 25°C and the supernatant was collected. The concentration of SC proteins in supernatant derived from nematodes at each stage was adjusted to 2.1 µg/ml.

GEL ELECTROPHORESIS AND MEMBRANE TRANSFER

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 5-20% gradient gels (e-PAGEL; ATTO) in a standard minislab PAGE apparatus (model AE-6500; ATTO). Full-Range Rainbow Molecular Weight Markers (GE Healthcare) were used to determine the molecular mass of the proteins. The proteins were either silver-stained using Sil-Best-staining kit (Nakalai Tesque) or transferred to Hybond-P PVDF membrane (Amersham). Silver staining of the gels was performed according to the manufacturer's instructions. The proteins were transferred to a PVDF membrane using a semi-dry transfer apparatus with transfer buffer (25 mM Tris,

pH 8.3; 192 mM glycine; 20% (v/v) methanol).

LECTIN BLOT ANALYSIS

Two horseradish peroxidase (HRP)-conjugated lectins (Seikagaku Corp.), ConA and WGA, were used for lectin blot analysis. After washing three times with 0.05% Tween-20-containing 10 mM Tris-buffered saline (TBST, pH 7.4), the membrane was blocked with 3% BSA-TBST for 2 h. The membrane was then washed three times with TBST, and incubated with 3 µg/ml HRP-conjugated lectin in 1% BSA-TBST for 1 h at room temperature. After washing three times with TBST, the proteins reactive to HRP-conjugated lectins were visualized by using a Konica immunostaining HRP-1000 (Konica).

Results

LECTIN LABELING

Among the five lectins examined, only WGA showed stage-specific differences

in binding (Table 2); WGA-binding was observed only to the outer surfaces of J3s and to the egg shells (Fig. 1A-E, H). Also, such bindings were detected at higher frequency in virulent isolates (S10, T-4, Ka-4 and Troia) than in avirulent isolates (OKD-1 and C14-5) (Fig. 1C, F).

A greater variety of lectins bound to the nematode eggs than to the other nematode developmental stages. However, neither DJ_{III} nor DJ_{IV} were labeled with any of the lectins tested. All lectins tested bound to PWN eggs, but significant differences were observed among isolates. Among the lectins ConA, WGA and RCA_{120} showed particularily strong labeling. These three lectins markedly differed in the labeling patterns of egg shells: while the labeling pattern of ConA was uniform (Fig. 1G), WGA showed a localized labeling on the egg shells (Fig. 1H). Only RCA_{120} exhibited two labeling patterns, uniformed or localized labeling (Fig. 1I, J).

The lectin-bindings to the outer surface of nematodes were disturbed when lectins were pre-incubated with their respective reactive sugars. This shows that all the lectins used in the present study definitely bound specifically.

PERIODATE PRETREATMENT

Oxidation of sugar moieties by periodate treatment had a great influence on WGA- and ConA-binding (Fig. 1K, L). The binding of WGA to the outer surface of nematodes was markedly increased by periodate treatment irrespective of the nematode stages (Fig. 1K). The binding of ConA also increased, although fluorescence was weaker than that of WGA (Fig. 1L). However, no significant change was observed in the bindings of PNA, UEA I and RCA_{120} after periodate treatment.

PROFILE OF SURFACE COAT PROTEINS ON SDS-PAGE

Typical patterns on SDS-PAGE of the SC proteins which derived from all PWNs stages except for the dispersal stages are presented in Fig. 2. Multiple protein bands, of 12.5 to 260 kDa, were detected in all lanes regardless of nematode stage. Some of them appeared only in specific stages, though most of them were common to all stages. For example, the protein of 35 kDa was detected in the SC protein of J2 and eggs, but not in that of J3, J4 and adults. By contrast, proteins of 37 kDa and 71 kDa appeared in SC protein of J3, J4 and adult, and not in that of J2 or eggs.

LABELING OF GLYCOPROTEINS BY LECTIN BLOT ANALYSIS

The results of the lectin bindings to the SC protein are shown in Fig. 3. Multiple glycoprotein bands were blotted by WGA in all nematode stages (Fig. 3A). In particular, 15 clear bands of glycoprotein of molecular weight from 11.5 to 275 kDa appeared in the extract from egg shells (Fig. 3A, lane 6). Of these, 9 glycoprotein bands of 11.5 to 39 kDa were common to all stages. ConA-blotting also generated various bands of glycoprotein ranging from 11.5 to 275 kDa in the extract of egg shells (Fig. 3B), of which only one weak band of 220 kDa was common to all stages.

Discussion

The present study detected the stage-specific differences in the SC of the PWN, and the molecular weights and glycosylation patterns of the PWN SC proteins were characterized. In the lectin-labeling study, WGA bound only to the 3rd-stage propagative juveniles except for eggs (Table 2, Fig. 1A-E). When the nematodes were pretreated with sodium periodate which cleaves carbon-carbon bond with vicinal hydroxyl groups of carbohydrates without altering the peptide structure (Woodward *et al.*, 1985), the binding of WGA to the outer surface of the nematode body was observed

in all stages (Fig. 1K). As well, several WGA-bound bands were detected in all the samples of SC proteins of PWN irrespective of nematode life stage in lectin blot analysis (Fig. 3A). These results suggest that the periodate pretreatment exposed WGA-binding sites on the surface of the nematode, which had been masked by carbohydrates, and thus allowed WGA access to its binding sites. The binding of ConA to the SC of the PWN was also increased by periodate pretreatment, although its fluorescence was weaker compared to that of WGA-binding (Fig. 1L). In lectin blot analysis, all the nematodes, regardless of life stage, shared the common glycoprotein of 220 kDa which bound to ConA in their SC (Fig. 3B). Therefore, the bindings of ConA would be disturbed by the obstacle of carbohydrate as well as those of WGA. Spiegel and McClure (1991) reported that periodate pretreatment of *Anguina tritici* exposed glycosyl residues of glycans whose conformation otherwise masks lectin binding sites, and suggested that these binding sites were probably subsurface. This seems consistent with our study, in which the results of the lectin-binding patterns after pretreatment of nematodes with sodium periodate corresponded to those of lectin blotting.

Most lectins examined showed strong binding to the PWN eggs (Table 2, Fig. 1G-J), indicating that they secrete and display glycoproteins modified with various glycans on their cuticle. The cross section images obtained by the confocal

laser-scanning microscopy clearly showed these glycans localized the outer surface of egg shells (data not shown). Lectin blot analysis also showed that the eggs displayed multiple glycoproteins with various glycans on their egg shells. Furthermore, each of the lectins tested showed a different pattern and location on the egg shells. The glycoproteins which had the glycans recognized by ConA uniformly appeared on the egg shell (Fig. 1G), and those which had the glycans recognized by WGA were localized on the egg shell (Fig. 1H). As for the expression patterns of the glycans recognized by RCA_{120}, there were remarkable differences between individuals (Fig. 1I, J). The patch reactivity with RCA_{120} on the egg shells may indicate that the glycans recognized by RCA_{120} were secreted onto the outer surface of eggs. It is known that protein glycosylation is important in protein folding, development, protein-protein interactions, immune response, host-pathogen interactions, and so on (Varki, 1993). The eggs have such various glycans which are not present in the other life stages of the PWN and therefore this may have some importance for the egg physiology and development.

WGA-binding was observed at higher frequency in virulent isolates than in avirulent isolates, although the number of isolates examined were limited indeed (Table 2). Fukushige & Futai (1985) also reported that the carbohydrates of egg shell were

different between pathogenic *B. xylophilus* and non-pathogenic *B. mucronatus*. In addition, the intensity of WGA-binding to the egg shell of *B. xylophilus* was higher than that of *B. mucronatus*. Based on these results, it is quite likely that either the glycoprotein or glycan, or both, recognized by WGA have an important role in the symptom development in the host-parasite interaction of pine wilt disease. Further studies are needed to determine whether these glycosylated molecules present in the SC of PWN are involved in pathogenesis.

Regarding the dispersal forms, *i.e.*, DJ_{III} and DJ_{IV}, of PWN, no lectin-binding was observed irrespective of nematode isolates (Table 2). This observation is worthy of futher study since DJ_{IV} is the special stage to enter the tracheal system of its vector beetle and then healthy pine tree, its host. It is known that SC of nematodes may play a role in evading host recognition (Gravato-Nobre *et al*., 1999). Similarly, the SC of DJ_{IV} of PWN might have a role in avoiding host recognition when the nematode invades the host pine tree.

SDS-PAGE revealed that the SCs of PWN developmental stages were different not only in carbohydrate patterns, but also in the protein. The change of carbohydrate patterns of SC with nematode development has been described for a variety of nematode species (Zuckerman & Kahane, 1983; Spiegel & McClure, 1991), and it is

known that this dynamic property of surface molecule expression during development is a strategy to avoid recognition by the host plant, and to evade the adhesion of their parasites (e.g., bacteria, nematophagous fungi) or other microorganisms (Spiegel & McClure, 1995). Therefore, full understanding of the characters of SC of PWN and their dynamic property would contribute to developing the novel biological control strategies for PWN.

Khoo (2001) described that the dynamic host-parasite interaction involves developmentally-regulated expression of specific glycan structures which must act as foreign antigens or subvert endogenous physiological signals. This concept would be applicable in the plant-nematode interaction. It is known that most plant lectins do not target plant carbohydrates but preferentially bind to foreign glycans (Peumans *et al*., 2000). Among *Pinus* spp., a lectin which had a high affinity to *N*-acetyl-D-glucosamine moieties was isolated from European black pine, *Pinus nigra*, and proved to have a similar binding specificity to WGA (Nahálková *et al*., 2001). In addition, considering our finding here that the SC of PWN had a high affinity to WGA, it seems quite probable that the early recognition step between host pine tree and the PWN during initial contact occurs through lectin-glycan interactions. Thus, the SC proteins of PWN should be of importance in pine–PWN interactions. The fundamental information on the

characters of the SC of PWN described in this paper will help to guide future studies on the relevance of SC of PWN to the pathogenic mechanisms of pine wilt.

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Table 1. *Lectins used and their respective competitive sugars.*

Lectin	Inhibiting sugar
Canavalia ensiformis agglutinin (ConA)	200 mM metyl-D-mannoside
Triticum vulgaris agglutinin (WGA)	200 mM *N*-acetyl-D-glucosamine
Arachis hypogaea agglutinin (PNA)	200 mM D-galactose
Ulex europaeus agglutinin (UEA I)	200 mM L-fucose
Ricinus communis agglutinin (RCA_{120})	200 mM D-galactose

Table 2. *Surface labeling of* Bursaphelenchus xylophilus *at different stages with FITC-conjugated lectins.*

PWN Stage	Isolate	Label lectins[1)]				
		ConA	WGA	PNA	UEA I	RCA_{120}
J2	S10	±	±	-	-	-
	T-4	±	±	-	-	-
	Troia	±	±	-	-	-
	OKD-1	±	±	-	-	-
	C14-5	±	±	-	-	-
J3	S10	±	+++	-	-	-
	T-4	±	+++	-	-	-
	Troia	±	+++	-	-	-
	OKD-1	±	+	-	-	-
	C14-5	±	±	-	-	-
J4 Male (Female)	S10	+	- (±)	-	-	-
	T-4	+	- (±)	-	-	-
	Troia	±	- (±)	-	-	-
	OKD-1	±	-	-	-	-
	C14-5	±	-	-	-	-
Adult Male (Female)	S10	±	- (±)	-	-	-
	T-4	±	-	-	-	-
	Troia	±	±	-	-	-
	OKD-1	±	-	-	-	-
	C14-5	±	-	-	-	-
Egg	S10	++	++	+	±	±
	T-4	+++	++	+	-	+
	Troia	+++	+	+	+	+++
	OKD-1	+++	±	+	-	+++
	C14-5	+++	±	±	-	+++
DJ_{III}	S10	-	-	-	-	-
	T-4	-	-	-	-	-
	Troia	-	-	-	-	-
	OKD-1	-	-	-	-	-
	C14-5	-	-	-	-	-

	S10	-	-	-	-	-
	T-4	-	-	-	-	-
DJ_{IV}	Troia	-	-	-	-	-
	OKD-1	-	-	-	-	-
	C14-5	-	-	-	-	-

[1)] Thirty nematodes at each stage were examined to determine if the cuticle was labeled with each lectin. The degree of surface labeling of the nematodes was classified according to the proportion of the number of nematodes labeled: –, 0/30; ±, 1/30 to 9/30; +, 10/30 to 19/30; ++, 20/30 to 25/30; +++, 26/30 to 30/30.

Fig. 1.

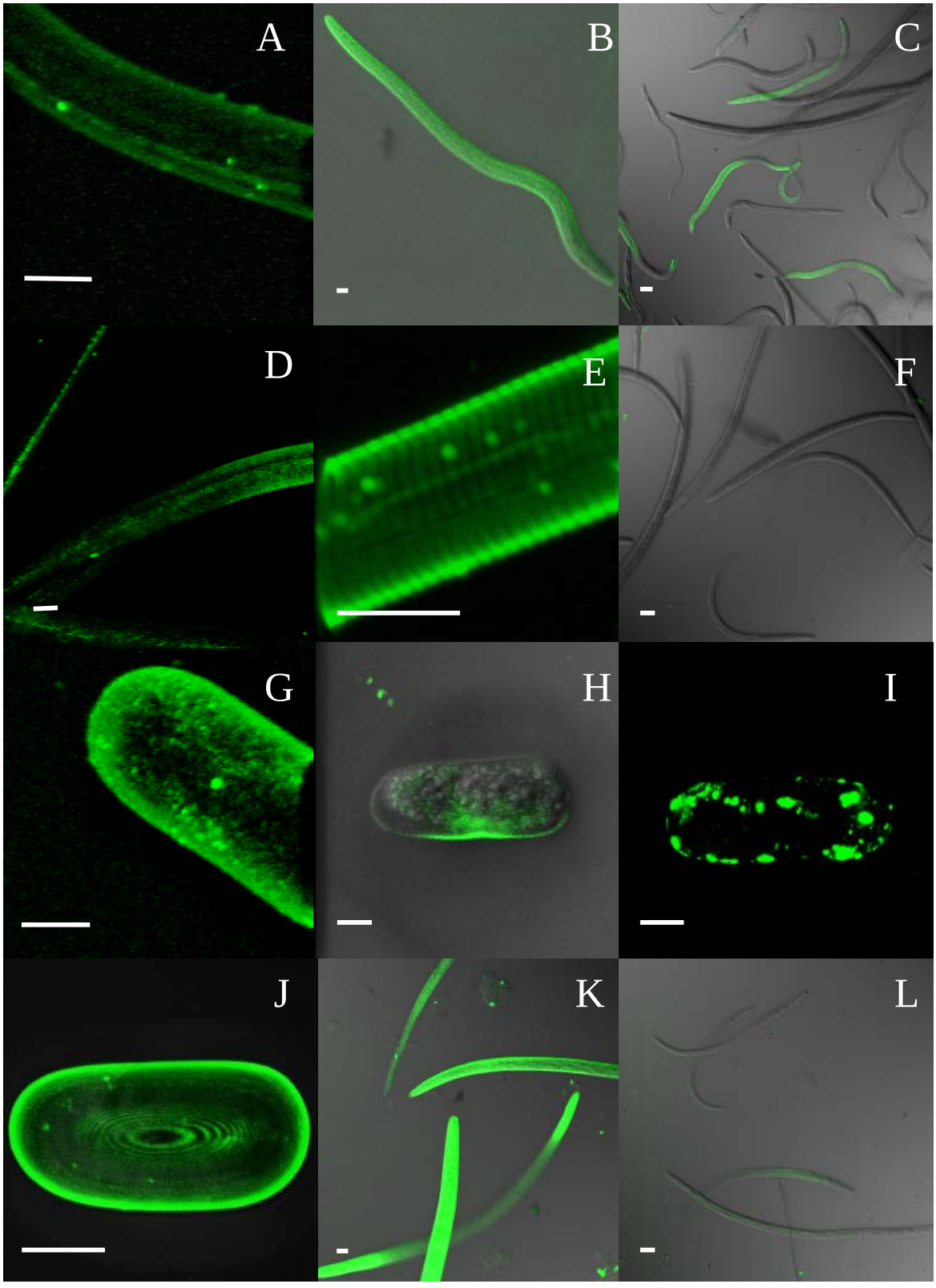

Fig. 2.

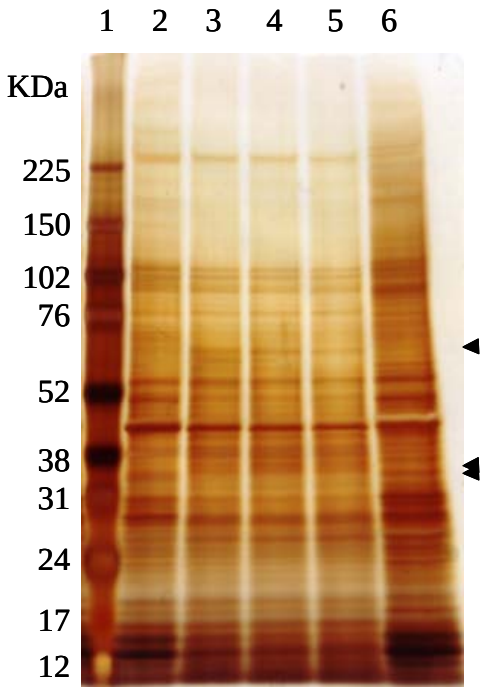

Fig. 3.

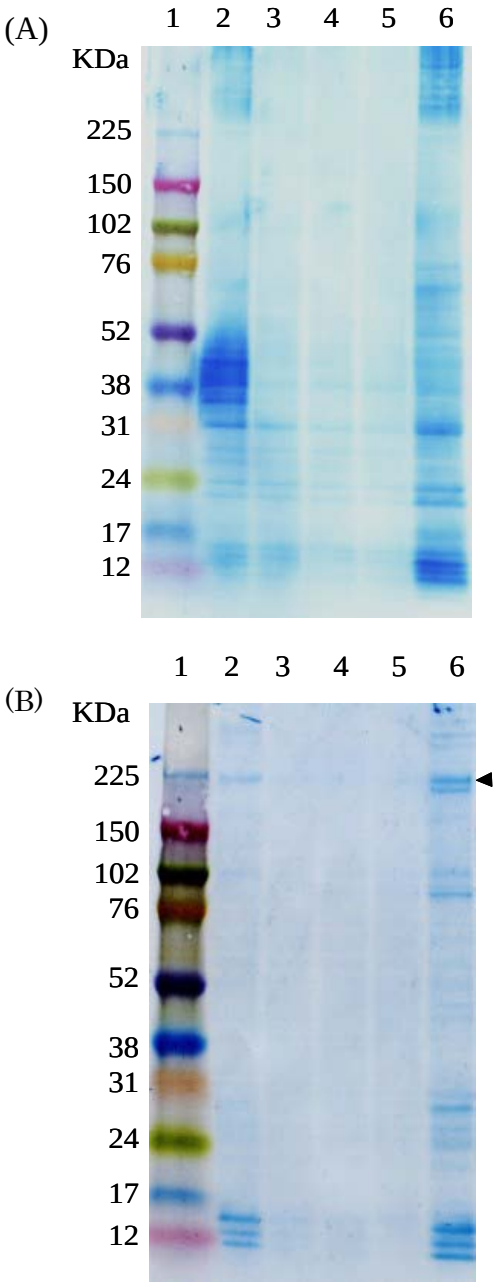

Fig. 1. *Surface fluorescence patterns of* Bursaphelenchus xylophilus *labeled with FITC-conjugated lectins. 3rd-stage juvenile (J3) of* B. xylophilus *S10 isolate (A, B), mixed stages of S10 isolate (C), J3 of Troia isolate (D, E), and mixed-stage nematodes of C14-5 isolate labeled with WGA (F); egg of C14-5 isolate labeled with Con A (G), eggs of S10 isolate labeled with WGA (H) and with* RCA_{120} *(I, J); mixed-stage nematodes of S10 isolate pretreated with 10 mM sodium periodate (*$NaIO_4$*) and then labeled with WGA (K) and with Con A (L). Scale bar:A, B, D, E, G, H, I, J, K = 10 µm; C, F, L = 20 µm.*

Fig. 2. *Typical protein band patterns on SDS-PAGE of surface coat proteins derived from different stages of* Bursaphelenchus xylophilus. *Proteins were visualized by silver staining. Lane 1, molecular weight standards; lane 2, surface extract of 2nd-stage juveniles; lane 3, surface extract of 3rd-stage juveniles; lane 4, surface extract of 4th-stage juveniles; lane 5, surface extract of adults; lane 6, surface extract of eggs.*

Fig. 3. *Typical glycoprotein band patterns of surface coat proteins derived from different stages of* Bursaphelenchus xylophilus, *blotted with HRP-conjugated WGA (A) or ConA (B). Lane 1, molecular weight standards; lane 2, surface extract of 2nd-stage*

juveniles; lane 3, surface extract of 3rd-stage juveniles; lane 4, surface extract of 4th-stage juveniles; lane 5, surface extract of adults; Lane 6, surface extract of eggs.